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Polysaccharide-based Freestanding Multilayered Membranes exhibiting Reversible Switchable Properties

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The design of self-standing multilayered structured based on biopolymers has been attracting increasing interest due to their potential in biomedical field. However, their use has been limited due to their gel-like properties. Herein, we report the combination of covalent and ionic cross-linking, using natural and non-cytotoxic cross-linkers, such as genipin and calcium chloride (CaCl₂). Combining both cross-linking types the mechanical properties of the multilayers increased and the water uptake ability decreased. The ionic cross-linking of multilayered chitosan (CHI)-alginate (ALG) films led to freestanding membranes with multiple interesting properties, such as: improved mechanical strength, calcium-induced adhesion and shape memory ability. The use of CaCl₂ also offered the possibility of reversible switch all of these properties by simple immersion in a chelate solution. We attribute the switch-ability of the mechanical properties, shape memory ability and the propensity for induced-adhesion to the ionic cross-linking of the multilayers. These findings suggested the potential of the developed polysaccharide freestanding membranes in a plethora of research fields, including in biomedical and biotechnological fields.

Introduction

Since the introduction of polyelectrolyte multilayers (PEMs) by Decher and co-worker, layer-by-layer (LbL) is the most popular and flexible approach to create functional ultrathin films with an unprecedented control at the nanometer and micrometer scales. 1-3 The great strength of this technique is based on the easy tailoring of physicochemical properties of the PEMs by simple adjustment of assembly and post-assembly parameters such as: temperature, concentration of polyelectrolytes, pH, charge density and molecular weight of polyelectrolytes, ionic strength and chemical or physical cross-linking. 4-7 Additionally, when compared with other techniques, LbL methodology allows that the different components in adjacent layers interact with each other attractively, helping to prevent phase separation and demixing. 8
This methodology enables the alternate deposition of complementary multivalent molecules (polyelectrolytes, dendrimers, proteins and nanoparticles) on virtually any type of substrate via a multimode of intermolecular interactions. 1, 7, 9, 10 For LbL based on electrostatic interactions, PEMs can be obtained through sequential adsorption of oppositely charge polyelectrolytes onto a myriad of substrates, which allow the development of innovative and complex materials. 7, 9, 11, 12 Firstly reported by Mamedov and Kotov 13 the release of thin multilayered films from a solid substrate to a free standing state with the use of a sacrificial layer, presented a tremendous potential when compared with their solid supported equivalents. Nowadays, using low surface energy substrates freestanding films could be simply obtained in mild conditions by simple detachment of multilayers from the template. 14-17 Among the polyelectrolytes that have been used chitosan (CHI) and alginate (ALG) received particular attention. These two polysaccharides share a marine origin and show potential to be assembled using LbL methodology. Such polysaccharides have been widely used due to their stability in physiological conditions and also to impart biological properties to the construct such as: biocompatibility, adhesiveness, bacteriostatic, fungicidal, antimicrobial and hemostatic activities. 18-21 Besides the stability of CHI-ALG membranes when compared with other polysaccharide films, their high water uptake ability and low stiffness impair cell adhesion. 14, 16, 22, 23 A common approach used to stiff these multilayers consists in the post-assembly treatment with cross-linking agents such as glutaraldehyde, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide in combination with N-hydroxysulfo-succinimide.
(EDC/NHs) and genipin.\textsuperscript{16, 24} Genipin is naturally derived chemical from gardenia fruit that has been extensively investigated in the cross-linking of amine-containing polymers\textsuperscript{25, 26} and multilayered films\textsuperscript{15, 18, 24, 27}. In CHI-based materials this cross-linker acts through a nucleophilic attack of CHI C-2 to C-3 genipin, resulting in opening of the hydroxypyrany ring and the formation of a nitrogen-iridoid which produce aromatic intermediates.\textsuperscript{28-30} Subsequent steps may involve radical-induce polymerization that create genipin heterocyclic conjugates which can be exploited to further tailor the properties of the final construct. Additionally, the ester groups of genipin can react with amino groups in CHI and secondary amide linkages can be stabilized\textsuperscript{28-30} (see scheme 1). This covalent cross-linking is permanent and present high selectively.\textsuperscript{31-33} The main advantages of genipin when compared with other covalent cross-linker agents is its low cytotoxicity and anti-inflammatory effects \textit{in vivo}.\textsuperscript{16, 26, 34} When CHIT/ALG films are cross-linked with genipin, since ALG does not contain primary amines, genipin will give rise to semi-interpenetrating polymer networks with free ALG chains entrapped inside cross-linked CHIT multilayers, as previously reported in other multilayered systems.\textsuperscript{27} To further improve the multi-properties of these multilayered films we propose to combine covalent and ionic cross-linking mechanisms. Using this methodology it will be possible to further crosslink the ALG chains in order to have two entangled cross-linked networks, forming a full-interpenetrating polymer networks.\textsuperscript{35} An ALG chain is comprised by mannuronic and guluronic acid units. Paired guluronic units can accommodate divalent ions in a shape of "egg box".\textsuperscript{36-39} Thus, ionic bonds between guluronic blocks in adjacent ALG chains can occur through ionic interactions between its carboxylic moieties and divalent calcium cations present in external medium (see scheme 1).\textsuperscript{36-41} This ionic cross-linking is reversible in the presence of strong calcium-coordinating ligands, such as ethylenediaminetetraacetic acid (EDTA).\textsuperscript{37, 39} To the data there are few reports of mixing covalent and ionic cross-linking in multilayered systems built by electrostatic interactions.\textsuperscript{42} Scheme 1 represents the different interactions within these multilayers upon combining both cross-linking mechanisms. The current study provides insight into the use of these membranes for a wide range of biomedical applications, since it will involve the characterization of the switch-ability of their water uptake, mechanical strength, as well as their calcium-induced adhesion propensity and shape memory ability upon ionic and/or covalent cross-linking.

Results and discussion

Morphology and composition

CHI and ALG were sequentially adsorbed on the surface of hydrophobic polypropylene substrates to produce robust, compliant and flexible membranes, which allowed their shaping in virtually any shape, as previously reported.\textsuperscript{14, 15, 22} The multilayers were mainly assembled due to electrostatic interactions between the charged groups. However, other types of interaction may play an important role on that such as the hydrophobic, hydrogen bonds and also chain entanglements.\textsuperscript{43-46} Thus, the multilayers can be easily detached from the substrate without the use of any post-assembly processes due to weak nature of the van der Waals forces between the initial layer (CHI layer) and the substrate.\textsuperscript{14, 17} This possibility gives rise to freestanding films, which opened the door to the direct experimental determination of several physicochemical and mechanical properties without the influence of the substrate.

In the present work, the membranes were cross-linked with genipin and CaCl\textsubscript{2} combining a covalent and an ionic cross-linking. The genipin cross-linking has the ability to amine-containing polymers, generating a greenish color on the samples.\textsuperscript{29, 47} An ionic cross-linking was also performed due to the ability of ALG to have reversible gelling properties upon ionic interactions with divalent ions such as calcium and the carboxylic acid moieties on the guluronic acid residues of ALG.\textsuperscript{38-40} CaCl\textsubscript{2} was used to induce ionic cross-linking in control membranes, namely CHI-ALG membranes with and without previous genipin cross-linking. The reversibility of the process was assessed with EDTA as chelating agent, i.e. with ability to "sequester" calcium ions.\textsuperscript{43, 48, 49}

The morphology of the developed freestanding membranes was evaluated by SEM (Figure 1). The results revealed an uniform deposition and free-defects multilayers which indicates the efficacy of the multilayers detachment using these supports. The SEM analysis was made using the upper part of the membrane (ALG side) instead of the substrate side.
(CHI side). It has been previously reported that the substrate side (side in contact with the substrate prior to the detachment) is smoother.\textsuperscript{14}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig1.png}
\caption{Morphology of the upper side of the membrane. (A) SEM micrographs of membranes without and (B) with genipin cross-linking after exposure to CaCl\textsubscript{2} and EDTA solutions. The scale bars are 50 µm and 10 µm for the overview and higher magnification, respectively. Cross-section micrographs of the different membranes were also performed. The scale bar is 10 µm.}
\end{figure}

The results reveal an apparently similar morphology between the samples and an uniform distribution of the constituent components. However, the genipin cross-linked membranes present a smoother surface when compared with CHI-ALG membranes.\textsuperscript{14, 15, 18, 22} Upon genipin cross-linking the film presents higher stiffness and lower water content which in turns led to lower roughness and produces more uniform films.\textsuperscript{18, 22-24, 27}

With the ionic cross-linking in the surface of the membranes appeared fibrillar-like structures. However, the roughness is not significantly changed (Figure 1). The calcium ions interact with deprotonated carboxylic groups of the guluronic unit of the ALG, leading to an ionotropic gelation of the ALG chain and gel-like structures.\textsuperscript{39, 50} We speculate that this mechanism upon drying might lead to the observed fibrillar-like structures on the surface of the membranes. With the use of EDTA the fibrillar-like structures disappeared since the divalent ions were removed from the multilayered system. In cross-linked membranes both, roughness and morphology, was not significantly changed in the presence of CaCl\textsubscript{2} neither of EDTA. This behavior can be assigned to the stabilizing effect of genipin in CHI-ALG multilayers.\textsuperscript{22, 23} All the membranes formulations are stable and easy handling, being these properties more pronounced after covalent and ionic cross-linking. Pioneer studies of Rubner and Shen et al. demonstrated that when the multilayers comprised a significant population of loops and tails upon drying a rough surface is observed, whereas a surface dominated by flat, train-like segments conducted to a smoother surface.\textsuperscript{51}

The membranes were also observed in cross-sections and the micrographs revealed an homogeneous morphology along the thickness and some porosity which could be beneficial to enhance nutrient transport to cells or to increase the surface area of the constructs. This diffusion ability of membranes based on CHI-ALG was already reported in previous studies.\textsuperscript{14, 15} The thickness of the freestanding membranes was also determined using cross-sections of all the formulations used (Figure 1). CHI-ALG membranes presented a dry thickness of 31.2 ± 2.4 µm or 28.9 ± 2.1 µm without and with genipin cross-linking respectively. These results corroborated earlier results, where cross-linking increased the stiffness of the multilayers without significant changes in PEMs’ thickness in the dry state.\textsuperscript{15} On the other side, the dry thickness with CaCl\textsubscript{2} cross-linking was about 36.8 ± 2.4 µm or 29.4 ± 2.2 µm for native and cross-linked membranes, respectively. Regarding, the use of EDTA increased the thickness of non-cross-linked membranes (42.4 ± 4.2 µm), but did not significant decreased the one in cross-linked membranes (25.7 ± 3.4 µm) probably due to the stabilizing effect of genipin cross-linking.

The presence of calcium ions in CHI-ALG membranes with and without genipin was confirmed by EDS (Figure 2). Additionally the reversibility of the ionic cross-linking using EDTA, as chelate agent, was also confirmed.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig2.png}
\caption{EDS spectra of the produced membranes.}
\end{figure}
Swelling Properties

Freestanding membranes based on CHI and ALG are well-known to be composed by hydrophilic groups such as amine, carboxyl and hydroxyl groups which can promote the water uptake. The freestanding membranes were cross-linked with genipin and the water-uptake decreased, due to the smaller free volume and intermolecular space between the polyelectrolyte chains, which limits the molecular mobility of the polymer chains at the nanoscale level. The swelling ability and thickness variation of the prepared membranes were evaluated by cyclic immersions in CaCl₂ and EDTA (Figure 3A and 3B). The use of CaCl₂ decreased the water uptake and the thickness variation due to the formation of stable complexes between ALG and divalent ions. However, using a competing ligand such as EDTA, the calcium was chelated and the cross-linking was reversed. Thus, the water uptake and thickness increased, being the values similar to the ones initially obtained when the membranes where immersed in the control medium (sodium acetate buffer with additional salt). These results are consistent with the fact that calcium-ALG based materials are stable within a wide range of pH values, being readily soluble in the presence of strong calcium-coordinating ligands, such as sodium EDTA. It is also important to point out that after the first set, the membranes are accommodated to the cyclic experiment and to the changes in the ion contents presents in the media environment. Additionally, besides the EDTA ability to remove the calcium ions the depletion is not 100% efficient. Thus the membranes may remain ionically cross-linked to some extent, which led to a more stable behavior for the subsequent rounds.

The decrease of water-uptake was more pronounced using genipin as the cross-linking agent. This behavior was expected since the physicochemical properties are highly affected by different types of cross-linking mechanisms and cross-linking density. In ionic cross-linking the divalent cations are believed to bind solely to guluronate blocks of the ALG chains, as their structure allows a high degree of coordination of the divalent ions. On the other side, the chemical cross-linking with genipin leads to the formation of a permanent network (covalent bonds) by the nucleophilic attack of the amine groups to the cyano ester groups of CHI.

The results were further compared with individual set of experiments where all the membranes formulations were placed in different pHs environments (pH 5.5 and pH 7.4) (Figure 3C). As expected the water uptake was higher in all the cases for pH 7.4 than 5.5. At pH higher than the pKₐ of CHI (pKₐ(CHI) ≈ 6.5) the amine groups are progressively uncharged and the carboxylic groups are increasingly ionized (higher negative zeta potential values). Thus, the electrostatic repulsion between the negative charged groups of ALG would increase gradually as the pH increases, leading to higher water uptake. Finally, comparing both types of cross-linking the individual set of experiments confirmed the results obtained in the cyclic ones. The same trend of cyclic experiments was also verified when the membranes were immersed in an EDTA solution. Altogether, these results confirmed the switch-ability of the ionically cross-linked multilayers.

The mechanical properties of freestanding membranes were evaluated in the wet state using an Universal Mechanical Testing machine. Figure 4A shows representative stress-strain curves for all the freestanding membranes formulations. The results show an increase of Young modulus (E), ultimate tensile strength (σₑₓₘₐₓ) and a decrease of the maximum extension (εₑₓₘₐₓ) with the genipin cross-linking (Figure 4B-4D). The same behavior has been previously reported when CHI-ALG membranes were cross-linked with genipin. Additionally, the CHI-ALG membranes were ionic cross-linked with CaCl₂ and an increase in the E and σₑₓₘₐₓ occurred accompanied by a decrease in the εₑₓₘₐₓ. On the other side, for membranes cross-linked with genipin the addition of CaCl₂ led to an increase in the maximum extension. Upon immersion of ionic cross-linked membranes in EDTA the mechanical properties were almost fully recovered which corroborated the reversible switchable properties of these membranes. In literature it has been reported that the mechanical behavior of ionic and covalent cross-linking is completely different. The stress applied to ionically cross-linked materials led to a relaxation of the multilayers, water release, and a plastic deformation, as the cross-link dissociate. While in covalently cross-linked ones the water is also release, leading to stress relaxation but there is an inability to dissociate and reform bonds which leads to significant elastic deformation.
In this work, online dynamic mechanical analysis (DMA) experiments were also performed on freestanding multilayered films to monitor the ionic cross-linking reaction in situ, as well as to follow-up the chelating action of EDTA. The values of storage modulus ($E'$) and loss factor ($\tan \delta$) are shown as a function of time (Figure 5).

Native and genipin cross-linked freestanding membranes immersed in CaCl$_2$ presented a continuously increase in $E'$ accompanied by a decrease on $\tan \delta$. In both cases the increase occurs within the first hours of incubation (1.5 hours = for native membranes and 2 hours = for genipin cross-linked membranes). The increase in $E'$ indicated the stiffening effect generated by the ionic cross-linker. The $\tan \delta$ is the ratio between the energy lost by viscous mechanisms and the energy stored in the elastic component, providing information about the damping properties of the material. The decrease in $\tan \delta$ indicates that the membranes acquire more elastic properties during cross-linking accompanied by a release of water molecules. The increase of $E'$ occurred due to the efficient ionic cross-linking of the multilayers and not to presence of counterions presents in the sodium acetate buffer with additional salt (0.15M). Post-assembly variations of the external media affect the behavior of multilayers, as shown in previous work. However, it has also been shown that online experiments with CHI-ALG membranes immersed in acetate buffer the $E'$ and $\tan \delta$ remain stable with time. The online experiments with EDTA medium also confirmed the chelate effect of this compound since the $E'$ decreased and the $\tan \delta$ increased. Altogether these results confirm the hypothesis that ionic cross-linking of PEMs further increased the mechanical properties of the freestanding membranes and this behavior can be easily reversed by an EDTA solution.

**Shape memory**

The shape memory effects pave the way toward the development of novel materials-based devices due to their demonstrated ability to undergo geometric transformation upon exposure to environmental stimuli, such as temperature, electrical, electromagnetic, light, humidity and hydration. A shape memory construct has basically the ability to be deformed by application of an external stress and fixed in a second shape, usually called as temporary shape. The water or solvent-driven shape recovery effect of freestanding membranes with and without genipin cross-linking was evaluated. Figure 6 shows representative photographs of water/solvent-triggered shape memory properties of CHI-ALG membranes with and without genipin cross-linking.
In both cases, membranes lifted off from the substrate presented a flat permanent shape that upon hydration can be virtually deformed in any shape. After elastic deformation, the helicoidal shape was fixed by dehydration, leading to a stable temporary shape. The dehydration step was performed with pure ethanol, allowing a fast extraction of plasticizing water molecules. The permanent shape of the membrane can be easily recovered by hydration, being the process completely reversible. This process is intrinsically related with the molecular mobility and glass transition dynamics of the two polysaccharides that are highly influence by the presence of water. A minimum hydration level to promote the shape memory recovery is strictly necessary, as previously demonstrated in online DMA experiments. Additionally the use of CaCl$_2$ as cross-linking offered a new pathway in shape memory ability and also the possibility to surpass the limitation imposed by the permanent shape, allowing us to have several permanent shapes. Using this methodology, freestanding membranes with and without genipin cross-linking acquired a new permanent shape (helicoidal shape) by hydration, deformation, winding around a mandrel and CaCl$_2$ cross-linking – see Figure 6. The dry CaCl$_2$ cross-linked membranes became rigid, and could not deform freely, keeping the helicoidal shape. The ionically cross-linked membranes were further deformed by hydration and its geometry was fixed by dehydration, leading to a new flat temporary shape. Upon rehydration the membranes became soft and easily deformed, recovering their initial helicoidal shape. The initial permanent shape 1 was also further recovered with the use of EDTA. Ethanol and CaCl$_2$ were used to stabilize the multilayers by chemical locking (Video S1 and S2). On the other side, water and EDTA solutions were used to unlocking them. From the best of our knowledge, it was the first time that reprogrammable shape was exploited based on reversible locked and unlocked with CaCl$_2$ and EDTA solutions. Altogether, the results confirm that CHI-ALG multilayers have shape-memory ability since they have the ability to memorize one or multiple temporary shapes and are able to return from this temporary shape to their permanent shape upon exposure to an external stimulus, such as the hydration one. These kinds of materials are ideal candidates for biomedical applications with minimal invasive procedures in which the temporary shape has to be preserved until the device is inserted in the defect/cavity to be filled, and then reach their application i.e. their permanent shape when a certain hydration level is achieved. The use of CaCl$_2$/EDTA is a sophisticate method to shape reprogrammed multilayers since both of them are cell friendly compounds, being calcium ions present in the physiological environment. The reprogramming with CaCl$_2$/EDTA is potentially amenable to other multilayered systems containing carboxylic groups. Overall, shape reprogrammable membranes are a sophisticated avenue to engineering complex forms with potential in biomedical field. Although, membrane-like devices were explored we envisage the applicability of such concept on other geometries, such as particles fibers or porous scaffolds.

**Calcium-induced adhesion**

In this work, the calcium-induced adhesion propensity of CHI-ALG membranes with and without genipin cross-linking was evaluated. To confirm the potential of our system we carried out a simple set-up experiment commonly used in these cases, in which the surfaces close to the edges of two wet membranes were overlapped and led to the formation of stable membranes with an overlap region. Afterwards, common tensile-strain tests were performed on such “overlapped membranes” to assess the efficiency of the methodology used. When carrying out the tests it was possible to conclude that the calcium-induced adhesion process was only efficient using CaCl$_2$, being the membranes stable for a period up to 1 month. Additionally, with this assay it was possible to conclude that genipin apparently inhibits the calcium-induced adhesion potential of these membranes, since the membranes fell apart within the overlap region (Figure 7A). For the native membranes overlap through CaCl$_2$ action, the $\sigma_{\text{max}}$, $\epsilon_{\text{max}}$ was comparable to the ones obtained in individual membranes (see Figure 7A-7C). However, the $\epsilon$ was relatively higher when compared with the individual set of experiments. All this parameters revealed the efficiency of the calcium-induced-adhesion process.

![Fig 7. (A): Representative images of freestanding membranes during a tensile test with and without genipin cross-linking upon overlapping in water-like media (CaCl$_2$ or sodium acetate-NaCl based solutions). (B): Representative Stress-Strain curves of overlapped CHI-ALG membranes by CaCl$_2$. (C): Ultimate Strain ($\sigma_{\text{max}}$) and Maximum Extension ($\epsilon_{\text{max}}$).](image-url)
further supported by the inefficient sticking of the native membranes while immersed in water. However, since the adhesion was considerable efficient we hypothesized that some interpenetration between the layers may have also occurred. To confirm that theory CHI-ALG membranes were overlapped with solvent casted membranes and an efficient adhesion occurred (Figure 8B). Regarding the inability of genipin cross-linked CHI-ALG membranes to stick while immersed in water and/or CaCl₂ solutions the main explanation could arise from the genipin cross-linking mechanism. Since genipin only reacts in amine containing polymers in CHI-ALG multilayers it will give rise to semi-interpenetrating networks with free ALG chains entrapped inside the CHI multilayers. The same behavior was previously reported for PLL based multilayers. Thus, the carboxylic groups of ALG will not be exposed in the surface of the two wet membranes which might hamper the ionic cross-linking and the calcium induced-adhesion process. Additionally, the reversibility of the induced-adhesion was also evaluated using EDTA. With the immersion in EDTA the membranes were weakly bonded in the overlapped area and readily detached (Figure 8B and 8C).

The calcium-induced adhesion mechanism revealed in this study can be used in other polyelectrolyte multilayers and these are expected to be useful for seal membranes around a bone defect, as well as, to use them as patches. Moreover, this methodology may be useful in the production of protecting layers and in biomaterials coatings. Using these approaches the process of damaging and “healing” can be repeated multiple times wherever and whenever it occurs, offering a new route toward safer and longer-lasting materials that would improve tissue-implant interfaces.

Conclusions

In summary we have demonstrated the successful possibility to combine covalent and ionic cross-linking towards the development of multifunctional multilayered freestanding membranes. Using this methodology it was possible to obtain a robust, compliant and flexible freestanding membrane whose architecture and properties can have an unprecedented control. The results showed that ionic cross-linking affects the mechanical properties and swelling ability of the membranes. Additionally, it also promotes calcium-induced adhesion and shape-memory ability. The ionic cross-linking was confirmed by EDS analysis, which revealed the presence of calcium in the membranes. In addition, the mechanical properties were also evaluated, and it was observed that the presence of calcium increases both stiffness and strength of the multilayered membranes. To further confirmed these results the ionic cross-linking was monitored in real time and revealed an increase of E’ with increasing reaction time. The chelate action of EDTA was also monitored in situ.

This study suggests that ionic cross-linking of multilayered CHI-ALG membranes deeply modulate outstanding properties of freestanding membranes such as mechanical strength, calcium induced-adhesion propensity and shape-memory ability. We believe that the reversible switch-ability of these properties by EDTA highlighted the potential of the developed freestanding membranes in a plethora of research fields.

Materials and methods

Production of freestanding membranes

CHI (Mw 190.000−310.000 Da, 82.6% degree of deacetylation, ref 448877, Sigma-Aldrich, USA) and low-viscosity ALG (538 kDa, ≈ 250 cP, ref 71238, Sigma-Aldrich, USA) were the two polyelectrolytes used for the construction of the multilayered films. CHI was purified by a series of filtration and precipitation steps both in water and ethanol. Freestanding membranes were produced using polypropylene supports previously washed in ethanol and water, using a protocol already reported. Briefly, polypropylene supports were alternately immersed in CHI and ALG polyelectrolyte solutions (0.2 % (w/v), pH 5.5, acetate buffer solution (0.1 M NaCl, pH 5.5) for 6 min each, with an intermediate washing step in acetate buffer solution in between (4 min). The procedure was repeated until 100 bilayers had been deposited on the templates. This process was performed using a home-made dipping robot specially designed for the automatic fabrication of multilayers. Afterwards, membranes were cross-linked with genipin (Wako chemical, USA) and/or with calcium chloride (VWR, USA). Briefly, for genipin cross-linking a solution (1 mg mL⁻¹) was prepared by dissolving the adequate amount of genipin into a dimethyl sulfoxide (Sigma-Aldrich, USA)/sodium acetate buffer (0.15 M NaCl, pH 5.5) mixture (1:4 (v/v)). CHI-ALG freestanding membranes were immersed in the cross-linking agent solution and incubated at 37°C overnight. Subsequently, membranes were thoroughly washed with ethanol and water to dissolve the amount of unreacted genipin, and left to dry at room temperature. For CaCl₂ cross-linking a solution of 100 mm was prepared in a sodium acetate buffer with additional salt (0.15 M NaCl, pH 5.5). CHI-ALG with and without chemical cross-linking were also immersed in CaCl₂ solution overnight. The
membranes were extensively washed in sodium acetate buffer and ultrapure water. To assess the reversibility of process (i.e. the depletion of calcium ions in the multilayered systems) the membranes cross-linked with CaCl₂ were immersed in EDTA solution overnight (0.05 M sodium acetate with 0.15 M NaCl pH 5.5).

Membranes morphology and composition
The surface of CHI-ALG membranes with and without CaCl₂ cross-linking was visualized by scanning electron microscopy (SEM). Membranes were gold sputtered and visualised using a Jeol JSM-6010LV microscope operating at an accelerating voltage of 15kV. In addition, energy dispersive spectroscopy (EDS) mapping was also performed. All samples were fixed by mutual conductive adhesive tape. Samples were analyzed in order to visualize the appearance of calcium component in the CHI-ALG membranes with or without exposure to EDTA solution. CHI-ALG membranes with or without genipin cross-linking were used as control.

Shape memory
All freestanding membranes with and without genipin cross-linking obtained by the LbL technique exhibited an initial flat permanent shape that upon hydration could be elastically deformed into an helicoidal/shape. Afterwards, the shape of the deformed membrane was fixed by dehydration with ethanol 100% in order to accelerate the extraction of water molecules. This stable shape (temporary shape) was retained until the membrane was hydrated again in water-like media. Additionally, the cross-linking reaction with CaCl₂ was also used to create a new permanent shape and consequently surpass the limitation imposed by the initial flat geometry of the fabricated membranes, permitting to obtain permanent shapes with other geometrical configurations. Upon immersion in an EDTA solution the membranes recovered their flat permanent shape 1 – see scheme 2.

Water Uptake
The water uptake ability of the freestanding membranes was measured by soaking dry films with known weight in sodium acetate buffer (pH 5.5). The swollen membranes were removed after 12 hours. The excess of solution was removed from the samples using filter papers (Filter Lab, Spain) and the freestanding membranes were weighed with an analytical balance (Denver Instrument, Germany). The water uptake was calculated as followed (equation 1):

\[
\text{Water uptake (%) } = \frac{W_w - W_d}{W_d} \times 100
\]

Where \(W_w\) and \(W_d\) are the weights of swollen and dried freestanding membranes, respectively.

Afterwards, the freestanding membranes were washed and immersed in a sodium acetate solution containing CaCl₂ (100 mM) for other 12 hours and the water uptake was determined, as aforementioned. Finally, to evaluate the reversibility of the process, the freestanding membranes were placed in an EDTA solution, during 12 hours, and the water uptake determined. The same procedure was repeated for cross-linked membranes with genipin. The cyclic experiment was further compared with individual set of experiments i.e. all the membrane formulations’ immersed in different media (sodium acetate solutions at pH 5.5 and 7.4).

The thickness of freestanding membranes during the cyclic experiments was also evaluated using a digital micrometer with a precision of 0.01 mm. The thickness variation (\(\Delta\) thickness) was calculated as followed:

\[
\Delta \text{Thickness (%) } = \frac{Th_w - Th_d}{Th_d} \times 100
\]

Where \(Th_w\) and \(Th_d\) are the thickness of swollen and dried freestanding membranes, respectively.

Mechanical Tests
Mechanical properties of freestanding membranes were studied in the wet state using an INSTRON 5540 (INSTRON Int., Ltd., High Wycombe, UK) universal testing machine with a cell load of 1 kN. Data presented is a result of at least three independent measurements. The dimensions of the specimens used were 60 mm in length, 6.5 mm in width, and 76.8 ± 8.2 \(\mu\)m (CHI-ALG), 65.7 ± 6.1 \(\mu\)m (CHI-ALG-CaCl₂), 79.8 ± 6.2 \(\mu\)m (CHI-ALG-CaCl₂-EDTA), 64.4 ± 9.1 \(\mu\)m (CHI-ALG-G), 61.4 ± 6.3 \(\mu\)m (CHI-ALG-G-CaCl₂-EDTA) in thickness. The load was placed midway between the supports with a span (L) of 10 mm. The crosshead speed was 1 \(\text{mm.min}^{-1}\). For each condition, the specimens were loaded until core break. Five samples were used per condition.

Online experiments were performed using dynamic mechanical analysis (DMA) to evaluate the mechanical/viscoelastic properties of the freestanding membranes during ionic cross-linking and calcium renewal with EDTA. Such assays were performed using a TRITEC2000B DMA from Triton Technology (UK), equipped with the tensile mode. The measurements were carried out at 37 °C and the distance between the clamps was 10 mm. Freestanding membranes were cut at about 5 mm width (measured accurately for each sample). The freestanding membranes were immersed in acetate buffer (0.15 M NaCl, pH 5.5) until equilibrium was reached. After measuring the geometry, the samples were clamped in the DMA apparatus and immersed in
a CaCl2 or in an EDTA solution. Tensile experiments were carried out at 1 Hz, with constant strain amplitude of 30 µm. A complete frequency scan was not performed in this study. In this case, the main goal was to evaluate the kinetic of the ionic cross-linking, using the same methodology previously performed for genipin cross-linking\textsuperscript{22} and for monitoring the mineralization process in situ. Additional 1 Hz is a standard frequency, which correspond to walking activities, and thus to normal activities.\textsuperscript{61,62} A static pre-load of 1 N was applied to keep the membrane tight. At least three samples were used for each condition with the same experimental settings; average values are presented.

**Calcium-induced adhesion experiments**

The freestanding membrane with or without genipin and CaCl2 cross-linking were cut in large rectangular strips (∼10 x 30 mm). Then, two strips of the same condition were put in contact with a small overlap of 5 mm. The contact was made between the two upper surfaces of the freestanding membrane. The assembly was dipped into a sodium acetate buffer containing CaCl2 and placed between two glass slides overnight. After that, the samples were removed from the glass slides and were placed in the universal testing machine (INSTRON Int., Ltd., High Wycombe, UK). Stripes of films were then tested with the same mechanical setup as described previously for universal testing machine. The reversibility of the calcium-induced adhesion was evaluated immersing the samples in an EDTA solution. To facilitate the visualization of the membranes safranin-O staining were used to promote a red color on them.

**Statistical analysis**

Statistical analysis was performed with Graphpad Prism version 5.0 for Windows (Graphpad software, USA). All variables are expressed as mean ± standard deviation (SD) from at least three independent experiments. Non-parametric (Kruskal – Wallis test) or parametric tests (one way ANOVA followed by Turkey test) depending on whether the samples from at least three independent experiments. Non-parametric variables are expressed as mean ± standard deviation (SD) for normally distributed populations or not, respectively.

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**Notes and references**


The combination of covalent and ionic cross-linking gives rise to synergetic effects in multiproperties of the freestanding membranes based on layer-by-layer methodology. The robustness, compliance, calcium induced-adhesion and shape memory ability will pave the way towards novel therapeutic applications in tissue engineering (TE) and biomedical applications.